

Remarks

Rejections under 35 U.S.C. §103

The Examiner has maintained the rejection of the claims as being obvious over Itokawa et al. in view of Horowitz et al. Applicants traverse this rejection and withdrawal thereof is respectfully requested. In maintaining the rejection, the Examiner raises the following points.

a) The Examiner asserts that reaching a level of inactivation of  $>10^4$  by contact with a cyclic lipopeptide at a concentration of 1-100  $\mu\text{M}$  is routine optimization of the method of Itokawa et al.

The present invention is drawn to a method of inactivating lipid-enveloped viruses in cell-free biological products using a cyclic lipopeptide, a salt of the lipopeptide, an ester of the lipopeptide or a mixture thereof at a concentration of 1-100 $\mu\text{M}$ . Itokawa et al. disclose that surfactins (1) and (2) have moderate anti-HIV activities in the XTT formazan assay for HIV-1 cytopathatic effects. See page 607, final paragraph preceding "Experimental" section. Itokawa et al. cite to reference No. 17 for the protocol details of the XTT formazan assay. A copy of reference 17, Weislow et al. J. Natl. Cancer Inst. 81:577-586 (1989) is attached hereto.

Weislow et al. teach that the XTT formazan assay may be used to evaluate a drug through its effects on the in vitro infection of cultured host cells by cell-free HIV-1 or by coculture with HB cells chronically infected with HIV-1. See Abstract of Weislow et al. As detailed on page 578, left column, first paragraph, final 3 lines, the XTT formazan assay detects drug-induced suppression of viral cytopathic effects by the generation of soluble formazan (XTT formazan) in the surviving cells. Thus, the bioassay of Itokawa et al. describes the moderate suppression of HIV-1 cytopathic effects on cells, and Itokawa et al. demonstrates that the drugs being tested act on virus replication in cell culture, not that the drugs are acting as virus inactivators that destroy the infectivity of products by killing the viruses, as achieved with the present invention.

There is no disclosure or suggestion in Itokawa et al. that by using the compounds of the presently claimed method all infectious particles in cell-free pharmaceutical products, e.g. proteins that are fractionated from blood or recombinantly produced, could be inactivated.

Contrary to the assertion of the Examiner, an optimization of Itokawa et al. will not result in the full inactivation of viruses in pharmaceutical products while maintaining the activity of the biological products, because Itokawa et al. has nothing to do with

inactivating, i.e. killing, viruses in cell-free products. There is no reasonable expectation of success for achieving a level of viral inactivation of  $>10^4$  using only 1-100 $\mu$ M of the cyclic lipopeptide at room temperature within 30 min. to 2 hours, from the teachings of Itokawa et al. Itokawa et al. pertains solely to the suppression of viral replication in cells.

Under MPEP §2144.05, Examiners are instructed that "a particular parameter must first be recognized as a result-effective variable, i.e. a variable which achieves a recognized result, before the determination of the optimum or workable ranges of said variable might be characterized as routine experimentation." Itokawa et al. is irrelevant to the present invention, which pertains to cell-free biological products. As shown in the attached article by the inventors, Vollenbroich et al., *Biologicals* 25: 289-297 (1997), the present invention functions through the direct killing of viral particles through the disruption of the viral membrane and capsid. Itokawa et al. uses surfactins for inhibition of viral replication. As such, none of the parameters disclosed in Itokawa et al. would be recognized as a result-effective variable for viral killing in non-cellular biological products and achieving the present invention is not merely routine optimization.

b) As a second point, the Examiner asserts that the teaching of Itokawa et al. of moderate viral inactivation provides motivation to "optimize" the method for full inactivation. As discussed above, the assay used in Itokawa et al. reports suppression of viral replication with infected cells. There is no motivation provided in Itokawa et al. to develop the present method because the present invention is a method of directly killing virus particles in non-cellular products. As such, Itokawa et al. is irrelevant to the present invention and no motivation is provided from the reference to achieve the present invention.

The assay used by Itokawa et al. reports a moderate level of suppression of viral replication with infected cells. There is no suggestion or motivation from Itokawa et al. to achieve the presently claimed method of directly killing viral particles in biological products. Nor does the present invention represent a mere routine optimization of Itokawa et al., since the compounds are being used in a completely different way. Horowitz et al. further fails to teach or suggest the present invention and the present invention is not achieved by combining the teachings of Horowitz et al. with those of the present invention. As such, withdrawal of the rejection is respectfully requested.

The Examiner has further maintained the rejection of claims 1, 3, 9 and 10 as being obvious over Naruse et al. Applicants traverse this rejection and withdrawal thereof is respectfully requested. In response to Applicants' arguments, the Examiner raises the following points.

a) The Examiner asserts that Applicants' argument that the method of Naruse et al. utilizes inhibition of viral replication is unsupported. As noted previously, Naruse et al. disclose a cytotoxicity test. Naruse et al. demonstrate that pumilacidins are inhibitors of herpes simplex type 1. See Abstract. In Naruse et al., antiviral activity was assessed using plaque reduction and dye uptake assays with HSV-1-Vero cells. See pages 274-275, "biological activity." Table 4 of Naruse et al. summarizes the results of the assays. It is readily evident that Table 4 reports that pumilacidins inhibit the replication of HSV-1 because acyclovir is used as the control. Acyclovir is well-known in the art to be an inhibitor of viral replication. Thus, it is readily apparent from the disclosure of Naruse et al. that the method utilized by Naruse et al. is one of inhibition of viral replication, not the direct killing of virus particles, as achieved with the present method.

In addition, Naruse et al. specifically disclose in the Abstract and page 275, Table 5 and "H<sup>+</sup> and K<sup>+</sup> - ATPase Inhibitory

Activity" that pumilacidins A and B are inhibitors of the enzyme activity of  $H^+$  and  $K^+$  - ATPase. One skilled in the art would not predict from this teaching that cyclic lipopeptides could be used for the inactivation of viruses protein-containing biological products without negatively affecting the biological activity of the products. However, as evidenced by the attached data (Attachment 1), the present inventors have shown that after exposure of various commercially available blood proteins, such as factor II, Factor VIII and antithrombin, to 100  $\mu$ M surfactin for 12 hours at 22°C, the activity of the proteins remained 100%.

Naruse et al. disclose in Table 4, the  $ID_{50}$  (concentration for 50% inhibition of cytopathic effects of the non-treated control) and  $TD_{50}$  (toxic dose) for the pumilacidins and acyclovir. For example, acyclovir has an  $ID_{50}$  of 0.2  $\mu$ g/ml with a  $TD_{50}$  of >100  $\mu$ g/ml in the dye uptake assay. Pumilacidin A and B, on the other hand, have  $ID_{50}$ 's that are very similar to the  $TD_{50}$ 's, i.e. a low therapeutic index. This means that the concentrations of pumilacidins required by Naruse et al. for achieving antiviral effects are very close to the toxic concentration. Such a low therapeutic index (difference between the  $ID_{50}$  and  $TD_{50}$ ), would lead one skilled in the art away from using the compounds as antiviral compounds.

b) The Examiner further asserts that Applicants' arguments regarding direct versus indirect activation is irrelevant with the statement that "claiming of a new property for a known product does not impart patentability." The Examiner is incorrect in this statement. The legal principal upon which the Examiner appears to be relying is one of inherency. "Under the principles of inherency, if a structure in the prior art necessarily functions in accordance with the limitations of a process or method claim of an application, the claim is anticipated." In re King, 231 USPQ 136 (Fed. Cir. 1986). However, Naruse et al. cannot be inherently functioning in the same manner as the present invention because Naruse et al. does not use the compounds in the claimed method. In addition, the principle that "claiming of a new property for a previously known product does not impart patentability" is described in MPEP §2112.02 "Process of Use Claims - New and Unobvious Uses of Old Structures and Compositions May Be Patentable." However, this section of the MPEP pertains to "product" claims that recite an intended use, not "method" claims. This section of the MPEP cites to In re Hack, 114 USPQ 161 (Crt. Cust. & Pat. App.). The claim at issue in Hack recited, "A brazing alloy for use under conditions of elevated

temperatures...", i.e. a product claim, that recited an intended use, not a method claim as with the present invention.

c) Finally, the Examiner further asserts that Applicants' argument that the measurement of viral growth and multiplication is indicative of inhibition and not inactivation is not persuasive. The Examiner notes that measuring viral infectivity is the standard means of determining viral "inactivation" (effectiveness), regardless of whether the agent acts directly or through inhibition. As discussed above in point a), Naruse et al. specifically discloses the inhibition of viral replication by pumilacidins. The present invention, on the other hand, is a method of directly inactivating viruses. Thus, the present invention functions by a completely different means as the assays of Naruse et al. Naruse et al. specifically disclose a method of inhibiting viral replication. The present claims recite the direct inactivation of virus as a feature of the claimed method. As such, the Examiner is incorrect that the means by which the methods of Naruse et al. and the present method function is irrelevant.

The Examiner maintains the rejection of claims 2 and 14 over Itokawa et al. or Naruse et al. combined with Horowitz et al. and



the rejection of claims 8 and 11 over Itokawa et al. or Naruse et al. combined with Vater et al. As noted previously, Horowitz et al. and Vater et al. fail to teach the deficiencies of Itokawa et al. and Naruse et al. As such, it is not possible to achieve the present invention by combining the references and withdrawal of the rejection is respectfully requested.

**Rejections under 35 U.S.C. §112, second paragraph**

Claims 1-9, 13-15, 18 and 19 have been rejected under 35 U.S.C. §112, second paragraph as being indefinite. More specifically, claim 1 has been rejected for recitation of inactivation of a factor of  $>10^4$ . The Examiner asserts that a "unit" must be given for the measurement to have meaning. Applicants traverse this rejection and withdrawal thereof is respectfully requested.

As described in the specification on page 8, final paragraph, and Table 1, reference to viral inactivation at a level of  $>10^4$  indicates that the viral titre is reduced by a factor of  $>10^4$ . Since the level of viral reduction is measurement of the factor of inactivation, no units should be present. However, claim 1 has been amended for clarification to indicate that "viral titre is reduced by a factor of  $>10^4$ ." Withdrawal of the rejection is respectfully requested.

Claim 2 has been rejected with the assertion that the metes and bounds of "room temperature" are not known because the specification fails to disclose what is intended by "room temperature." Applicants traverse this rejection and withdrawal thereof is respectfully requested. "Room temperature" is a well-known and readily accepted term used in laboratory protocols. As evidence that "room temperature" is an accepted and defined term, attached hereto as Attachment 2, is an excerpt from the textbook, "Deutsches Arzneibuch, 9<sup>th</sup> Ed. (1986), wherein room temperature is defined as "15 to 25 °C." Withdrawal of the rejection is therefore respectfully requested.

Claim 18 has been further rejected for recitation of "blood products", "products from blood" and "biotechnological pharmaceutical products." Claim 18 has been amended to recite "products isolated from blood and biotechnological pharmaceutical products consisting of human proteins." With the amendment to claim 18, the claim recites two distinct groups of products, those which have been isolated and those which are biotechnological in nature, i.e. have been made recombinantly. As such, withdrawal of the rejection is respectfully requested.

Claim 19 has been rejected as being in improper Markush group form. Claim 19 has been amended to be in correct form. Withdrawal of the rejection is respectfully requested.

Rejections under 35 U.S.C. §112, first paragraph for lack of enablement

Claims 1-10, 13-15, 18 and 19 have been rejected under 35 U.S.C. §112, first paragraph for lack of enablement. More specifically, the Examiner asserts that the invention is not enabled for the preparation of products for in vivo administration. The Examiner asserts that the agents used in the present invention are known to be toxic and damaging to blood cells. Applicants traverse this rejection and withdrawal thereof is respectfully requested.

The present invention is drawn to a method of inactivating lipid-enveloped viruses in cell-free biological products. The present invention is not drawn to a method of administering products in vivo. As such, the rejection by the Examiner is not relevant and is inapplicable to the claimed subject matter. The question of enablement for the present invention only regards whether or not the virus may be inactivated in cell-free biological products by following the recited method and possibly whether such products retain their activity. The inventors have

clearly demonstrated the enablement of the claimed invention through the data of the specification and the data of Attachment 1. As such, the enablement of the invention has been clearly demonstrated and withdrawal of the rejection is respectfully requested.

Applicants further note that the specification clearly teaches on page 5, that, "as a result of the exceedingly low in vivo toxicity of the lipopeptides used according to the invention, these inactivating substances may also be allowed to remain in the pharmaceutical products at the above-mentioned concentrations." The specification also teaches that alternatively, "following inactivation, the lipopeptides employed may be removed from the products by reverse phase HPLC on a C18 column, or by adsorption chromatography on a silica gel column." Thus, the specification clearly instructs that the lipopeptides need not be removed or if removal is desired, means for doing so.

If any questions remain regarding the above matters, please contact Applicant's representative, MaryAnne Armstrong, PhD (Reg. No. 40,069), in the Washington metropolitan area at the phone number listed below.

Pursuant to 37 C.F.R. §§1.17 and 1.136(a), Applicants respectfully petition for a two (2) month extension of time for

filing a response in connection with the present application. The required fee of \$200.00 is attached hereto.

If necessary, the Commissioner is hereby authorized in this, concurrent, and future replies, to charge payment or credit any overpayment to Deposit Account No. 02-2448 for any additional fees required under 37 C.F.R. §§ 1.16 or 1.17; particularly, extension of time fees.

Respectfully submitted,

BIRCH, STEWART, KOLASCH & BIRCH, LLP

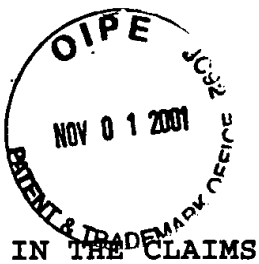
By Joe McKinney Muncy (Key No. 40,065)  
Joe McKinney Muncy #32,334

KM/MAA/

P.O. Box 747  
Falls Church, VA 22040-0747  
(703) 205-8000

consisting of human proteins.

19. (Amended) The method of claim 18, wherein the product isolated from blood is a vaccine ~~are selected from the group consisting of vaccines.~~



VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE CLAIMS

Claims 1, 18 and 19 have been amended as follows.

1. (Thrice Amended) A method of inactivating lipid-enveloped viruses in a cell-free biological product ~~non-cell culture biological products~~, which comprises

providing a cyclic lipopeptide, a salt of the lipopeptide, an ester of the lipopeptide, or a mixture thereof;

contacting said product with the cyclic lipopeptide, salt of the lipopeptide, ester of the lipopeptide, or mixture thereof as an inactivating agent, at room temperature for 30 minutes up to 2 hours,

wherein the agent is added to said product at a concentration of 1-100  $\mu\text{M}$  and viral titer is reduced by a factor of  $>10^4$  an ~~inactivation factor  $>10^4$  is achieved, wherein the agent directly inactivates~~ through the direct inactivation of any lipid-enveloped viruses present in said product by the agent.

18. (Amended) The method of claim 1, wherein the cell-free biological product is ~~non-cell culture biological products are~~ selected from the group consisting of ~~blood products~~, products isolated from blood, and biotechnological pharmaceutical products

# ARTICLES

reference 17  
of Holawa et al.  
(Chem. Pharm. Bull.  
42(3) 604-607  
(1994))

## New Soluble-Formazan Assay for HIV-1 Cytopathic Effects: Application to High-Flux Screening of Synthetic and Natural Products for AIDS-Antiviral Activity

Owen S. Weislow, Rebecca Kiser, Donald L. Fine, John Bader,  
Robert H. Shoemaker, Michael R. Boyd\*

We have developed an effective and optimally safe microculture method for rapid and convenient assay of the in vitro cytopathic effects of human immunodeficiency virus (HIV-1) on human lymphoblastoid or other suitable host cells. The assay procedure is applicable to the evaluation of drug effects on in vitro infections induced directly in cultured host cells by cell-free HIV-1 or by coculture with H9 cells chronically infected with HIV-1. The assay uses a newly developed tetrazolium reagent that is metabolically reduced by viable cells to yield a soluble, colored formazan product measurable by conventional colorimetric techniques. This simple microassay minimizes the number of plate manipulations typically required with other assay methods and, coupled with computerized data collection and analysis, facilitates large-scale screening of agents for potential antiviral activity. To support and enhance the discovery of new anti-HIV-1 agents, the National Cancer Institute is offering investigators worldwide the opportunity to submit new candidate agents for anti-HIV-1 screening with this method. [J Natl Cancer Inst 81:577-586, 1989]

Screening is an essential tool for new drug discovery. The use of a relatively simple in vitro assay was critical to the early identification of 3'-azido-3'-deoxythymidine (AZT), the first new antiviral drug with demonstrable therapeutic efficacy in acquired immunodeficiency syndrome (AIDS) (1,2). However, since the discovery of AZT, relatively few new nonnucleoside classes of compounds that interfere with replication and/or cytopathic effects of human immunodeficiency virus (HIV-1) have been discovered. Current antiviral screening strategies typically depend on analysis of viral antigen synthesis (3,4) or reverse transcriptase activity (5). Although these methods are sensitive, they involve relatively lengthy procedures and do not simultaneously provide a quantita-

tive measure of drug cytotoxicity that would allow estimation of an in vitro "therapeutic index." Techniques that do provide direct, quantitative determination of HIV-1-induced cytopathic effects typically require time-consuming macroscopic observation of plaque formation (6) or microscopic observation of cell viability (7,8) or syncytium formation (9,10). Consequently, these techniques may not be useful for large-scale screening of potential antiviral compounds.

As part of the response of the National Institutes of Health (NIH) to the AIDS epidemic, the Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute (NCI), has undertaken the development of a project for large-scale drug screening that would allow greatly expanded testing of synthetic and natural products for anti-HIV-1 activity (11). This screening program is intended to serve as a national resource, permitting scientists from academic or industrial settings worldwide to submit novel compounds for screening. The screen will also be used extensively to search for new potential anti-HIV-1 compounds from the NCI repositories of synthetic and natural

Received January 12, 1989; accepted January 23, 1989.

Supported in part by Public Health Service contract N01CO-74102 from the National Cancer Institute, National Institutes of Health, Department of Health and Human Services.

O. S. Weislow, R. Kiser, and D. L. Fine, Research Support Program, Program Resources, Inc., National Cancer Institute-Frederick Cancer Research Facility, Frederick, MD.

J. Bader, R. H. Shoemaker, and M. R. Boyd, Developmental Therapeutics Program, Division of Cancer Treatment, National Cancer Institute, National Institutes of Health, Bethesda, MD.

We thank Drs. R. Gallo, S. Broder, and B. Chabner for reviewing the manuscript.

\*Correspondence to: Michael R. Boyd, M.D., Ph.D., Office of the Associate Director for Developmental Therapeutics, Division of Cancer Treatment, National Cancer Institute, Executive Plaza North, Room 843, Bethesda, MD 20892.



products. Establishment of a high-flux drug screen with the potential to accommodate as many as 40,000 or more samples per year has required the development of a simple, automated method consistent with procedures for safe handling of HIV-1.

Assays based on the metabolic reduction of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) have been used to measure virus-induced cytopathic effects (12,13) and cell proliferation (14). The cellular reduction of MTT results in the formation of a colored, insoluble formazan product. For quantitative estimation of cell growth and viability, the formazan must be solubilized prior to colorimetric determination. For non-adherent cell preparations, centrifugation may also be required. We describe a new, simplified, tetrazolium-based assay that we have derived from methods recently developed for in vitro antitumor screening (15,16). This assay uses the new tetrazolium reagent 2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide (XTT) and cell lines sensitive to the lytic effects of replicating HIV-1. The assay detects drug-induced suppression of viral cytopathic effects by generation of a soluble formazan (XTT formazan) (fig. 1) in surviving cells.

## Materials and Methods

### Cells and Viruses

CEM-SS cells were obtained from Peter Nara (10); MT-2 cells transformed with HTLV-I (6), from Douglas Richmond; C3-44 cells transformed with HTLV-II, from William Mitchell (17); and LDV-7 cells, from Robert Gallo. The suitability of various T-lymphocyte-derived cell lines for inclusion in the microculture ~~XTT-antiviral assay~~ was initially determined on the basis of their sensitivity to the lytic effects of HIV-1 infection. Other criteria were the abilities of the cells to metabolically reduce XTT to measurable quantities of XTT formazan and to show increased XTT formazan production due to inhibition of virus infection by an agent. More recently, cell lines have also been evaluated for use on the basis of their relative ease of growth; for example, the ability to grow in the absence of interleukin-2. All cells are grown in RPMI-1640 medium plus 10% fetal calf serum with 50 µg of gentamicin/mL. To ensure consistency of target-cell sen-

sitivity to virus and drug, cells are routinely replaced every 2 months from frozen stocks. Similarly, virus-producing H9 cells used in the cocultivation (cell-to-cell) assay described here are discarded and replaced with freshly infected cells every 2 months.

Virus stocks were prepared in H9 cells. The viability of chronically infected H9 cell cultures, and their capacity to produce high virus titers were enhanced by (a) maintenance of the cultures in the exponential phase of growth and (b) periodic addition of log-phase, uninfected H9 cells. In the standard procedures for the maintenance of H9 cells, we used our modification of a protocol suggested by P. Nara (personal communication). Once a week, we divided cultures of H9 cells that were producing HIV-1 (III<sub>B</sub> or RF variants). We diluted stock cell cultures 1:5 or 1:10 to yield a final concentration of  $1 \times 10^5$  cells/mL. At the time the cultures were divided, one-fifth of the volume of infected cells was replaced with an equal volume of uninfected cells. Three days after the split, 20% of the medium was replaced with fresh medium, and 2 days after the addition of uninfected cells, virus-containing supernatants were harvested by centrifugation at 3,000 rpm for 15 minutes at 4 °C. Aliquots (1 mL) of the supernatants were frozen in liquid nitrogen for later use. We titrated virus stocks using a 4-day syncytium assay as previously described (10). Highly infectious, virus-producing H9 cells were maintained for the cocultivation assay by daily 50:50 dilution of the cultures described here in fresh medium. The syncytium assay of Nara et al. (10) was used for quantitation of the number of virus-producing cells in the infected H9 culture; 80%-90% of these cells were producing virus. Typical concentrations of infectious virus are  $3 \times 10^4$  syncytium-forming units (SFU)/mL for the III<sub>B</sub> variant of HIV-1 and  $5 \times 10^5$  SFU/mL for the RF variant.

Sensitivity to the lytic effects of HIV-1 infection was determined in preliminary experiments for each host cell line by titrations of cell-free virus or H9 cells chronically infected with HIV-1. For the cocultivation studies, the final VIC (quantity of virus-infected cells added) was the lowest number of infected cells that yielded at least 70% uniform suppression of XTT formazan production in host cells in 7 days. This was accomplished with a VIC of 400 infected H9 cells per well in CEM-SS or LDV-7 hosts and 100 infected H9 cells per well in MT-2 or C3-44 hosts. For the studies of cell-free virus infections, the amount of virus added

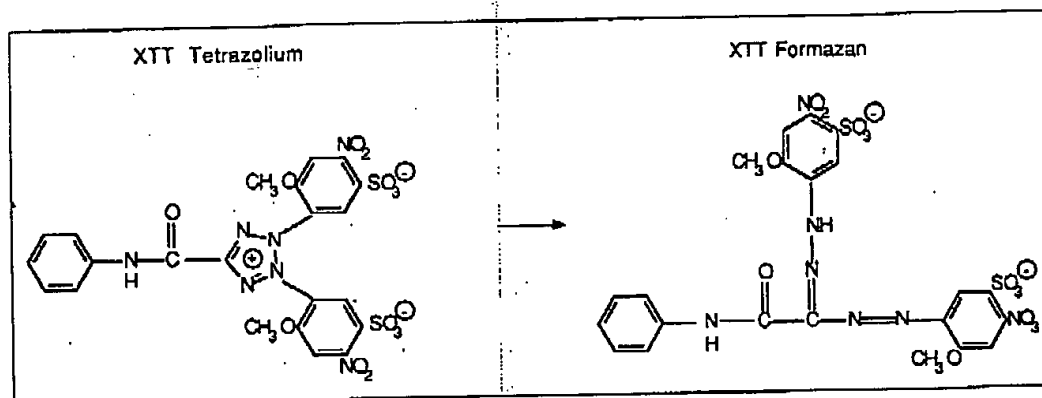


Figure 1. Chemical structures of XTT (colorless) and XTT formazan (orange).

was such that the final multiplicity of infection (MOI; ratio of number of infectious virus particles/number of target cells) was the lowest MOI yielding 70% suppression of XTT formazan production in host cells in 7 days. In the cell-free virus assays, the MOIs, which were established by syncytium analysis, were 0.1 for CEM-SS and LDV-7 hosts and 0.01 for MT-2 and C3-44 hosts.

### Drugs

All compounds were provided by the Developmental Therapeutics Program, Division of Cancer Treatment, NCI. Stock solutions were prepared in 100% dimethyl sulfoxide at the highest achievable concentration for each agent. Initial drug dilutions (1:400) resulted in a maximum culture concentration for dimethyl sulfoxide of 0.25%, which had no apparent direct toxic effects on the cell lines used or the HIV-1 infection. Compounds used in these studies included AZT; 2',3'-dideoxyadenosine; 2',3'-dideoxyinosine; 2',3'-dideoxycytidine; dextran sulfate; castanospermine; pepstatin; aurintricarboxylic acid; suramin; and cyclosporine.

### XTT Assay

We performed extensive evaluation of two antiviral screening protocols (fig. 2) derived from methods recently described for antitumor assays with XTT (15). Our protocols were developed from preliminary evaluations of cell growth characteristics in microtiter trays, virus sensitivities, and drug dilution protocols (data not shown). These initial studies were required to ensure that there would be sufficient time during the test (a) for the virus to kill the target cells or otherwise modulate cell growth, (b) for the anti-HIV-1 compound to be effective, and (c) for determination that the growth of the cells, and thus XTT formazan production, which is read as optical density, would not exceed the measurement limits for the photometers used.

Figure 2A illustrates the protocol that uses chronically infected H9 cells as the virus source. Infectivity of the H9 line was maintained by periodic dilution of the infected culture with uninfected log-phase H9 cells as described here. Uninfected host cells are mixed with different numbers of HIV-1-infected or uninfected H9 cells and dispensed in 100- $\mu$ L aliquots to appropriate wells of a microtiter tray containing 100- $\mu$ L dilutions of test compounds or media. Seven days after distribution of infected cells to the microtiter trays, we add to each well 50  $\mu$ L of a mixture of 1 mg of XTT/mL and 0.01-0.02 mM *N*-methylphenazonium methosulfate (15). Details of the synthesis of XTT have been reported previously (16), and the reagent is now commercially available (Polysciences, Warrington, PA). The trays are reincubated for an additional 4 hours to allow for XTT formazan production; their plastic covers are then replaced with adhesive plate sealers (Dynatech, Alexandria, VA), the contents of each plate are mixed, and optical densities are determined with a V-max photometer (Molecular Devices, Inc.) at a test wavelength of 450 nm and a reference wavelength of 650 nm. Uninfected cells or cells that are protected by drugs and have continued to proliferate produce the soluble orange XTT formazan (fig. 3A), and the cultures yield high optical densities (ODs). Cells not protected by drugs are killed by the virus and/or do not proliferate; they produce less XTT formazan and thus yield lower optical densities. Data are expressed as percent of XTT formazan from untreated control cells, as determined by the following equation: % of untreated control XTT formazan = (test OD/control cell OD)  $\times$  100.

Drugs are further compared on the basis of the concentration that increases XTT formazan production in infected cultures to 50% of that in untreated, uninfected control cells ( $EC_{50}$ ; protection in infected cells) or the concentration that inhibits XTT formazan production in uninfected cultures to 50% of that in untreated, uninfected control cells ( $IC_{50}$ ; cyto-

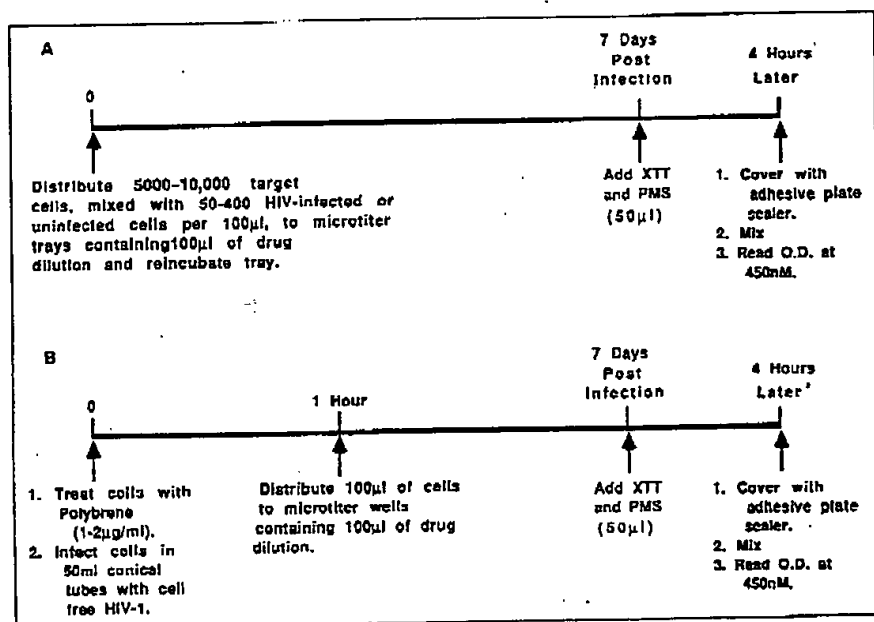


Figure 2. Time lines of critical events in drug-screening protocols.  $10^4$  target cells infected with cell-free virus (A) or 400 H9 cells infected with HIV-1 (B) are added to dilutions of test compounds in 96-well trays. Trays are incubated at 37  $^{\circ}$ C in air plus 5%  $CO_2$  for 7 days. A mixture of XTT and *N*-methylphenazonium methosulfate (PMS) is added at the end of this incubation, and plates are reincubated for 4 hr to permit color development (XTT formazan production) as described in Materials and Methods.

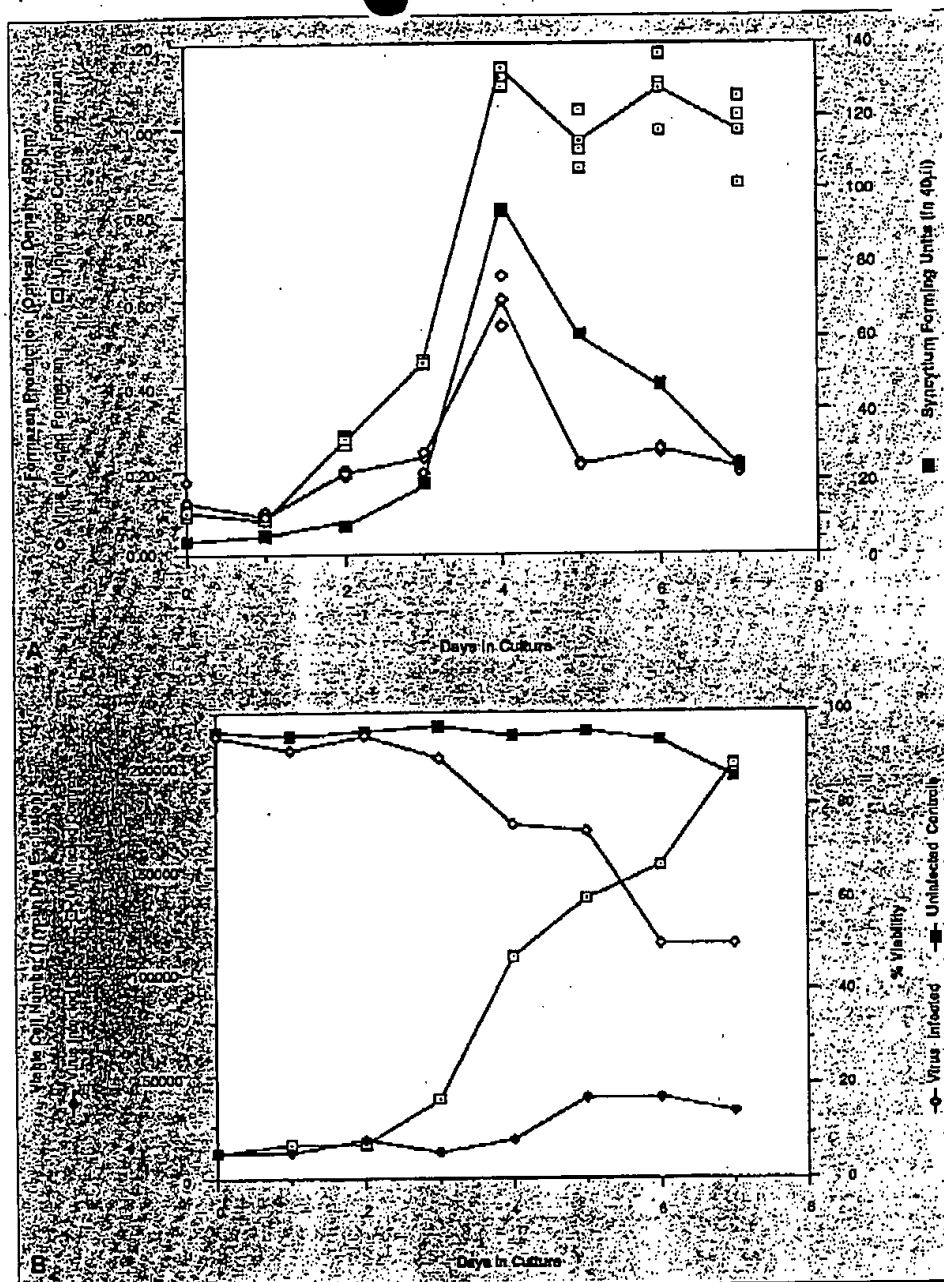


Figure 3. Relationships of XTT formazan production to cell viability and synthesis of infectious virus. CEM-SS cells ( $10^4$ ) were mixed with H9 cells (400/well) infected with HIV-1 ( $10^4$ ) or uninfected. Samples were taken from triplicate wells for assays of XTT formazan and infectious virus at 24-hr intervals for 7 days (A). Samples were also taken at these intervals for trypan blue dye exclusion analysis of cell viability (B) as described in Materials and Methods.

toxicity in uninfected cells). These calculations are performed by simple linear interpolation.

Figure 2B illustrates the procedures used for cell-free virus infection and drug screening. Following pretreatment with 1–2  $\mu$ g of hexadimethrine bromide (Polybrene)/mL, pelleted cells are incubated in 100–200  $\mu$ L of medium with cell-free virus for 1 hour in 50-mL conical centrifuge tubes at 37 °C in air plus 5% CO<sub>2</sub>. Infected cells are distributed in 100- $\mu$ L aliquots (containing  $10^4$  cells) to round-bottom microtiter trays containing, when required, 100  $\mu$ L of diluted test agents or media; they are then reincubated. After 7 days of incubation, the assay is completed as described for the cocultivation assay.

#### Syncytium and p24 Assays

We accomplished correlation of XTT formazan production with HIV-1 internal core p24 antigen synthesis using a p24 antigen-capture assay (Du Pont Co., Wilmington, DE). Culture fluids harvested from drug test plates or other sources were diluted 1:100 in 10% triton X-100 and stored frozen at –70 °C until they were required for assay. Aliquots (200  $\mu$ L) of the triton-treated samples were added in duplicate to microtiter wells previously coated with rabbit polyclonal anti-HIV-1 p24 serum and incubated at room temperature overnight. After washing and blotting, 100  $\mu$ L of biotinylated polyclonal anti-HIV-1 p24 was added to each of the

appropriate wells, and the plates were reincubated at 37 °C for 60 minutes. A solution of streptavidin-horseradish peroxidase was added after additional washing and blotting. The contents of each plate were mixed and reincubated for 15 minutes at room temperature and, after washing and blotting, the *o*-phenylenediamine dihydrochloride substrate was added in 100- $\mu$ L aliquots. Color was allowed to develop in the dark at room temperature for 30 minutes, and the reaction was halted by the addition of 4 N H<sub>2</sub>SO<sub>4</sub>. Optical density was measured at 490 nm, and the concentration of p24 was determined by comparison with a standard curve of known concentrations of p24.

The syncytium assay described by Nara et al. (10) was used for quantitation of infectious virus. Supernatants from test plates (40  $\mu$ L) were examined in CEM-SS cell monolayers at multiple dilutions to obtain countable levels of SFU (50–200 SFU/well) in 4 days. Data were expressed as SFU/mL. Cell-free virus stocks were also titrated by this method.

### Quality Controls

The screening of diverse compounds at the rate of 800 or more per week requires effective and efficient data accrual, storage, display, and analysis. Each screening test must be evaluated for uniformity and consistency with respect to appropriate quality controls. It is essential to promptly detect and correct problems that could have arisen in cell growth, virus-induced cytopathic effects, and positive controls. We have incorporated a number of quality control checks that permit computerized monitoring of all data for such potential aberrances. Manual overrides are available, and all data are briefly scanned by the laboratory staff to ensure that quality control standards are met. Although drugs are assayed in duplicate in the test plates, valid statistical data obtained in preliminary studies with three to six replicate wells per point indicate that well-to-well variation in the microculture XTT antiviral assay is generally  $\leq 10\%$ . As a quality control measure, plates are discarded if this variation is  $>25\%$ . Plates are also discarded if virus-induced cell killing decreases to  $<50\%$  or if there is less than a twofold increase in XTT formazan production above background in control cells. Even when there is a quality control problem, if available data clearly indicate that a drug is not effective, negative results are reported. An assay is also considered to be negative if microscopic observations appear to indicate drug-induced antiviral protection by XTT formazan production but fail to show the presence of intact cells in two assays. Negative assays that pass all quality control requirements are not routinely repeated. Assays are discarded if the positive control, AZT, which is included in at least one assay in a series, fails to show the expected degree of protection; all drug assays with questionable data on the AZT control are repeated.

## Results

### Initial Studies

Basic protocols were established for infection of target cells and drug treatment (fig. 2). Preliminary comparisons be-

tween cocultivation (cell-to-cell) (fig. 2A) and cell-free virus (fig. 2B) modes of infection showed similar levels of protection by AZT in MT-2 or CEM-SS cells (data not shown). Therefore, a majority of the initial feasibility studies used HIV-1 (III<sub>b</sub>) in the convenient cocultivation assay protocol with chronically infected H9 cells as the virus source.

Figures 3A and 3B illustrate the relationships among XTT formazan production, synthesis of infectious virus, and cell viability in a 7-day cocultivation assay. In this typical experiment, 10<sup>4</sup> CEM-SS target cells mixed with 400 infected or uninfected H9 cells were added in triplicate to wells of individual microtiter plates and assayed daily for XTT formazan production and numbers of viable cells (trypan dye exclusion) as described. Supernatant samples (50  $\mu$ L) were taken daily from infected cultures, frozen in vapor-phase N<sub>2</sub>, and subsequently tested as a group by syncytium assay for infectious virus. XTT formazan production began to plateau 4 days after the start of culture (fig. 3A). However, the total number of viable cells per milliliter in uninfected cultures, as determined by trypan blue dye exclusion (fig. 3B), continued to rise through day 7 of the culture despite a small decrease in the percentage of cell viability. The data suggest complex relationships among XTT formazan synthesis, cell viability, and virus synthesis in infected cell cultures. There were no evident differences in XTT formazan production in infected and uninfected cultures at the start of the incubation period (fig. 3A). XTT formazan synthesis in infected cultures appeared to be similar to that in uninfected controls from day 1 to about day 4, but on day 5 of culture, synthesis in the infected cultures decreased to approximately 20% of that in the uninfected controls.

The production of extracellular virus (fig. 3A) paralleled XTT formazan synthesis in infected cultures, while the number of viable cells per milliliter (fig. 3B) remained fairly steady through the 7-day period, rising to  $<5 \times 10^4$  cells per well by day 5, compared with  $135 \times 10^3$  cells per well in the uninfected controls. The percent of viable cells in infected cultures decreased to  $\approx 50\%$  by day 6. There appeared to be a continual increase in the synthesis of infectious virions (fig. 3A) that was independent of an apparent decrease in the number of viable host cells as measured by trypan blue dye exclusion. A possible basis for these apparent contradictions was revealed by microscopic observations of the cultures. Beginning 2 days after the start of culture, there was a gradual increase in the number of virus-induced multinucleated giant cells (syncytia), which resulted in a net decrease in the number of single, viable cells over time (data not shown). It is conceivable that, initially, the formation of syncytia caused an apparent decrease in cell viability (determined by trypan blue dye exclusion) without actually impacting virus synthesis. However, with time, HIV-1-induced cytopathic effects eventually result in the destruction of syncytia, suppression of XTT formazan production, and virion synthesis.

Data derived from these initial experiments also indicated that background absorbance levels in cell-free, reagent controls were unacceptably high (optical density, 0.3–0.4; data not shown). Subsequent analysis suggested that a common component of media, the pH indicator phenol red, might

be responsible for the high background optical densities observed. Thus, for all subsequent experimental work described here, phenol red was deleted from the culture medium.

#### Determination of Antiviral Activity

A typical test plate is illustrated in figure 4; in the plate shown, the CEM-SS cell line (10) is used as the target. In this example, the prototypical anti-HIV-1 agents 2',3'-dideoxyadenosine (wells B11, C11-B4, and C4) and AZT (wells D11, E11-D4, and E4) supported cell survival and thus significant XTT formazan production over a broad range of drug concentrations. The effects of various drug dilutions on uninfected target cells are also shown in figure 4; there is a single well for each drug dilution to demonstrate potential cytotoxicity (F11-F4 for 2',3'-dideoxyadenosine and G11-G4 for AZT).

#### Quantitation and Validation of Results

The quantitative data for AZT that were collected and plotted by computer are shown in figure 5. Drug comparisons are made on the basis of  $EC_{50}$  (protection in infected cells; fig. 5, bottom curve);  $IC_{50}$  (cytotoxicity in uninfected cells; fig. 5, top curve); and in vitro "therapeutic indices." In the example shown in figure 5, AZT has an  $EC_{50}$  of 0.186  $\mu M$ , an  $IC_{50}$  of 191  $\mu M$ , and an in vitro therapeutic index ( $IC_{50}/EC_{50}$ ) of 1,027.

The effects of AZT concentration on the relationships among XTT formazan production, HIV-1 p24 antigen synthesis, and infectious virus production [syncytium assay (10)] are shown in figure 6. As a result of increased cellular survival, there was a dose-related increase in XTT formazan production with increasing AZT concentrations. This was followed by reduction in XTT formazan production at the highest concentrations of AZT, as a result of drug cytotoxic-

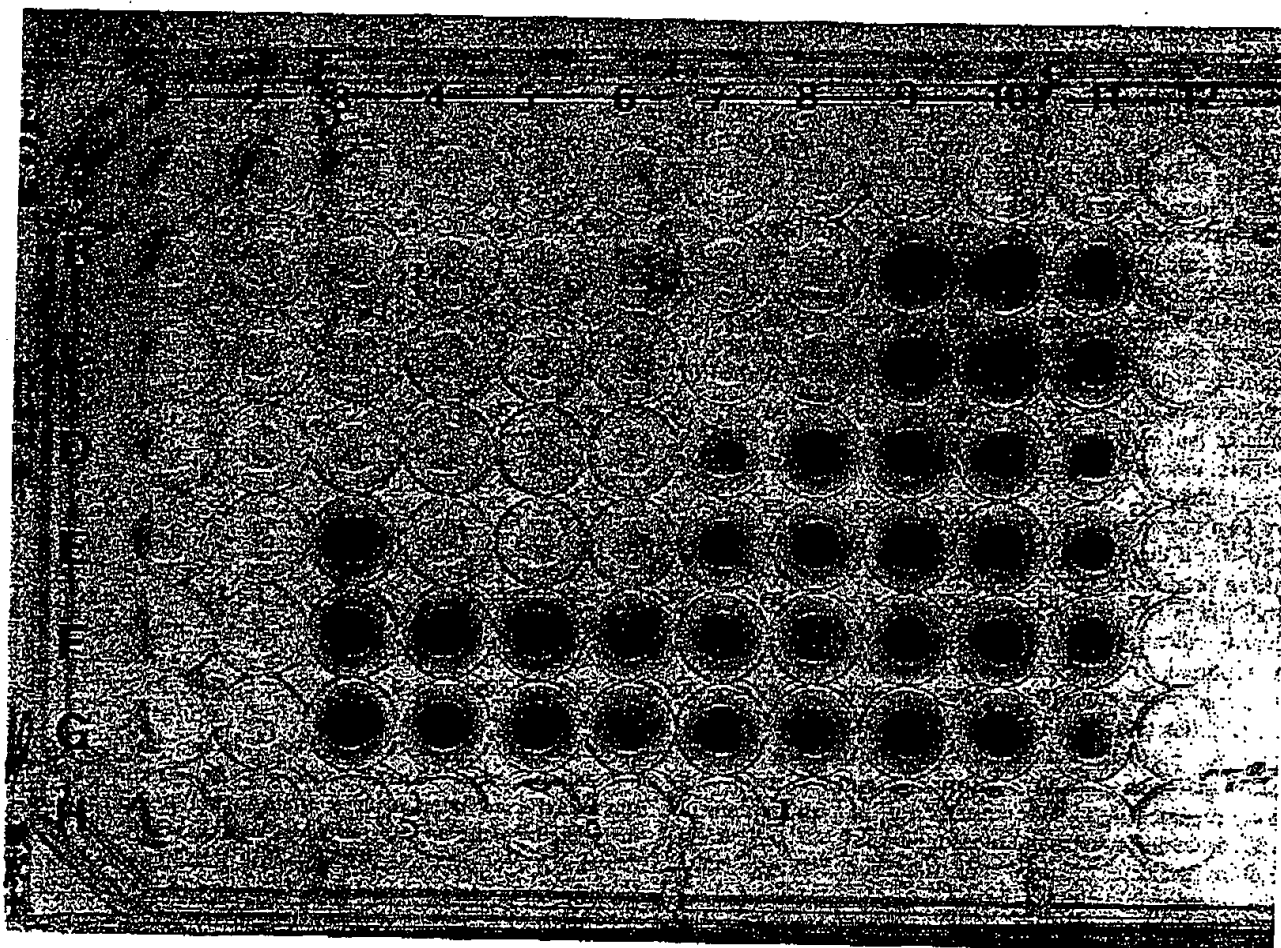


Figure 4. Typical test plate at time of assay end point. In this 7-day assay, CEM-SS cells ( $10^6$ ) infected by cocultivation with H9 cells (400/well) infected with HIV-1 (III<sub>2</sub>) were tested against AZT and 2',3'-dideoxyadenosine (DDA). Medium free of phenol red was used throughout procedures to minimize background optical density. B2-G2: reagent control (XTT-PMS in media). B3-D3: untreated, infected control cells. E3-G3: untreated, uninfected control cells. B11, C11-B4, and C4: duplicate 10-fold dilutions of DDA (starting at 500  $\mu M$ ) against infected CEM-SS target cells. D11, E11-D4, and E4: duplicate 10-fold dilutions of AZT (starting at 1 mM) against infected CEM-SS target cells. F11-F4: single-well dilutions of DDA directed against uninfected, control CEM-SS cells. G11-G4: single-well dilutions of AZT directed against uninfected control CEM-SS cells. A11-A4: single-well dilutions of DDA without cells. H11-H4: single-well dilutions of AZT without cells. Orange color indicates formazan product of XTT reduction by viable cells.

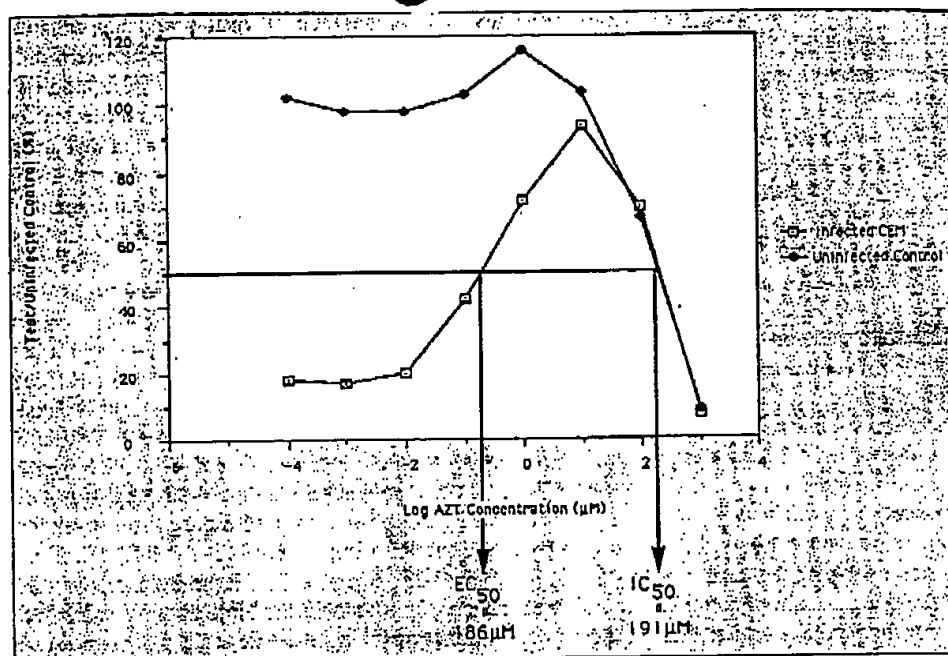


Figure 5. Quantitation of XTT formazan production in AZT-treated cultures of CEM-SS cells. Infected or uninfected CEM-SS target cells ( $10^4$ /well) treated with 10-fold, serial dilutions of AZT.  $EC_{50}$  represents concentration of AZT (e.g.,  $0.186 \mu M$ ) that increases (protects) XTT formazan production in infected cultures to 50% of that in untreated, uninfected control cells.  $IC_{50}$  represents inhibitory or toxic concentration of AZT (e.g.,  $191 \mu M$ ) that reduces XTT formazan production in uninfected cultures to 50% of that in untreated, uninfected control cells. Levels of XTT formazan in untreated, infected control cells were 20% of those in untreated, uninfected control cells.

ity. Cell viability, as determined by trypan blue dye exclusion, paralleled the changes in XTT formazan production (data not shown). At the same time, p24 synthesis and infectious virus production (SFU) were suppressed by AZT, relative to synthesis and production in untreated, infected control cells. This suppression was also dose dependent. Similar quantitation of drug cytotoxicity is not readily obtainable by p24 antigen analysis, and syncytium assay gives only semiquantitative results.

#### Effects on Cell Lines

To date, the drug screen has been conducted predominantly with two cell lines, CEM-SS (10) and MT-2 (6). Selected antiviral compounds were tested in two additional T-lymphoblastoid cell lines, C3-44 and LDV-7 (table 1). The assays in each cell line were optimized for killing of target cells by HIV-1-infected H9 cells in preliminary titration experiments. The number of cocultivated, infected H9 cells was

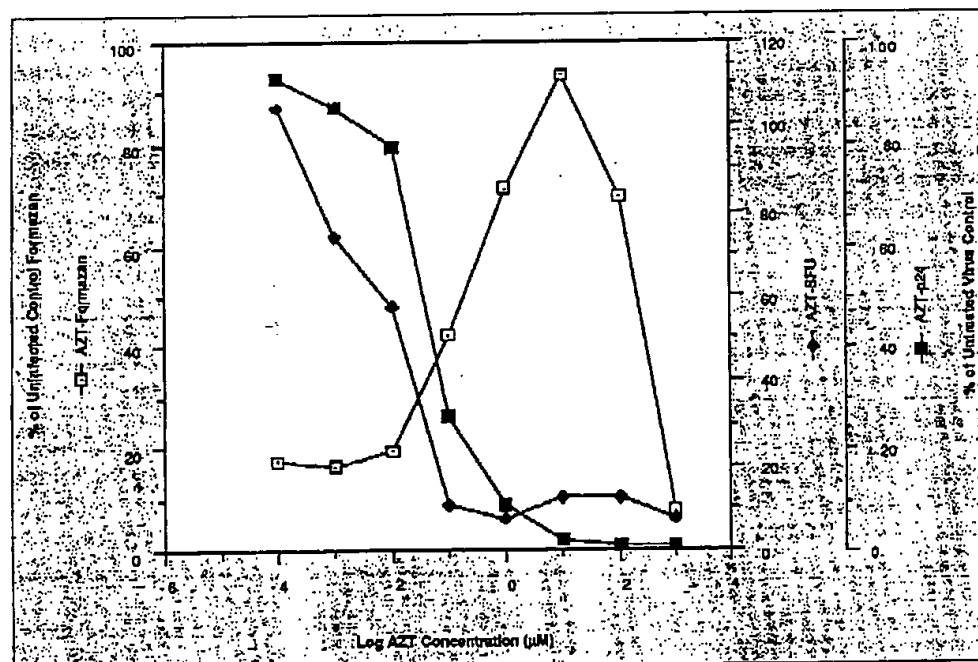


Figure 6. Correlation of XTT formazan production by infected, AZT-treated CEM-SS target cells with production of infectious virus as measured by syncytium assay and p24 antigen synthesis as determined by p24 antigen-capture ELISA (73).

**Table 1.** Comparison of T-lymphoblastoid cell line sensitivities with protection induced by various antiviral agents as determined by XTT formazan production in cocultivation protocol

Drug*	Cell (EC <sub>50</sub> in $\mu$ M)†			
	CEM-SS	MT-2	LDV-7	C3-44
AZT	0.2	0.012	0.05	0.007
DDA	7	1	1	2
Cyclosporine	13	6	>167	25
Pepstatin	380	>728	>728	618
Castanospermine	201	>1,100	1,015	123
ATA	35	16	57	14
Suramin analogue	28	29	41	17

\*DDA = 2',3'-dideoxyadenosine, ATA = aurantricarboxylic acid.

†EC<sub>50</sub> = concentration of drug at which infected cells produce level of XTT formazan 50% of that produced by uninfected control cells.

determined so that, in each target cell culture, the XTT formazan synthesis in 7 days would be reduced at least 70%. The test drugs were selected because of their demonstrated anti-HIV-1 activities in other test systems and for known or apparent differences in their mechanisms of action. It is clear from these data that not all of the T-lymphoblastoid host cells potentially usable in the in vitro assay system are uniformly protected by known antiviral compounds.

#### Cell-Free Virus and Cocultivation Protocols

Although a majority of our initial feasibility studies for the XTT-based anti-HIV-1 assay were conducted with the cocultivation protocol, the daily use of this assay in a high-flux screen would require an unattainably high degree of consistency in the maintenance of both target cells and chronically infected H9 cells. A constant level of infectious virus production from the chronically infected H9 cells would be mandatory to ensure a consistent MOI input on a day-to-day basis. This requirement would be essential for assay standardization; it would provide for uniform killing of infected controls and consistency of drug-induced protection from experiment to experiment. Although the cocultivation protocol is relatively convenient and seemed otherwise potentially attractive for use in a high-flux screen, we were unable to obtain the necessary day-to-day consistency and assay standardization using this mode of virus infection. Moreover, it was not possible to precisely quantitate MOI with the cocul-

tivation assay. The percentage of virus-producing cells appeared to fluctuate daily, and levels of virus-induced killing varied, sometimes by as much as 100%-200%. In addition, other laboratories (18) have recently reported marked differences between cocultivation and cell-free virus assays in the levels of protection induced by individual compounds and in the ability of a given assay to identify a protective agent.

The III<sub>b</sub> variant of HIV-1, which we used initially in the cell-free virus protocol, produced infectious virus titers of 10<sup>3</sup>-10<sup>4</sup> SFU/mL from chronically infected H9 cells. Scale-up from small feasibility studies to large-scale screening with the cell-free virus protocol (fig. 2B) was not practical with these virus preparations. On the other hand, H9 cells chronically infected with the RF variant of HIV-1 consistently produced high infectious virus titers (>10<sup>5</sup> SFU/mL). MOIs necessary to provide a consistent 70%-80% reduction of XTT formazan synthesis in the 7-day cell-free virus assay were easily established in preliminary titration experiments by use of the RF variant with MT-2 (MOI, 0.005) and CEM-SS (MOI, 0.05) target cells. Large volumes of these virus preparations have been divided into aliquots and frozen; these preparations are being used in scale-up operations in the high-flux screen with use of the cell-free virus protocol (fig. 2B). They were also used in the experiments comparing results of the cell-free virus and cocultivation protocols using MT-2 and CEM-SS target cells (table 2), which are described later in this report; these results further highlight the advantages of the cell-free virus protocol over the cocultivation protocol.

Initially, we used 10<sup>4</sup> target host cells per microtiter well in the cocultivation protocol (fig. 2A). This culture condition may contribute to the plateau of XTT formazan production and premature termination of infectious virus synthesis in the absence of drug treatment (fig. 3A). Because this approach weighs the assay heavily in favor of apparent drug-induced protection, it may generate unnecessarily large numbers of false-positive results. In preliminary cell-free virus studies (data not shown), it was determined that a lower number of host cells (5 × 10<sup>3</sup> per well) would permit a continued increase in XTT formazan production and infectious virus synthesis over the 7-day culture period. Thus we used this number of host target cells per well in all subsequent cocultivation and cell-free virus assays.

As we have noted, initial evaluations suggested no signif-

**Table 2.** Comparison of EC<sub>50</sub> and IC<sub>50</sub> in cell-free virus and cocultivation protocols in MT-2 and CEM-SS cells\*

Drug	MT-2 cells				CEM-SS cells			
	EC <sub>50</sub> ( $\mu$ M)		IC <sub>50</sub> ( $\mu$ M)		EC <sub>50</sub> ( $\mu$ M)		IC <sub>50</sub> ( $\mu$ M)	
	CFV	Cocultivation	CFV	Cocultivation	CFV	Cocultivation	CFV	Cocultivation
AZT	0.43	0.24	>1,000	>1,000	0.05	0.65	>1,000	>1,000
DDC	2.68	NO	87.20	3.23	0.33	0.26	144.00	30.30
DDA	0.00	1.64	>500	>500	0.00	13.90	>500	>500
DDI	20.20	15.10	>625	>625	3.82	10.20	>625	>625
Dextran sulfate	0.12	5.34	>39.5	>39.5	0.03	0.95	>39.5	>39.5

\*HIV-1 (RF) was used for all cell-free virus (CFV) assays. MOI was 0.005 for MT-2 cells and 0.05 for CEM-SS cells. Cocultivation protocol used 10<sup>2</sup> H9 cells chronically infected with HIV-1 (III<sub>b</sub>) to infect 10<sup>4</sup> MT-2 target cells; 400 infected H9 cells were used to infect 10<sup>4</sup> CEM-SS target cells. DDC = 2',3'-dideoxycytidine; DDA = 2',3'-dideoxyadenosine; DDI = 2',3'-dideoxyinosine; NO = not obtainable.



icant differences in the sensitivities of the two assay procedures to drug-induced, antiviral protection. These initial studies were conducted with a limited number of nucleoside analogues in MT-2 cells. The data in table 2 suggest that, depending on the cell line and the compound tested, the method of testing could have a significant impact on assay results. The cell-free virus assay appears to provide a more sensitive system for identifying protective compounds. These results appear to be independent of drug mechanisms, as can be seen from a comparison of the effects of two of the drugs used with the two assay protocols: 2',3'-dideoxyadenosine, a nucleoside analogue (a reverse transcriptase inhibitor), and dextran sulfate, which is purported to function at the level of virus attachment (19,20). Comparison of the EC<sub>50</sub> values in table 2 shows that the cell-free virus assay indicates much more potent protection by dextran sulfate than that indicated by the cocultivation procedure, depending on the cell line used.

We observed differences between the cytotoxic effects of the test compounds on MT-2 and CEM-SS cell lines. The agent 2',3'-dideoxycytidine appeared to be more toxic in MT-2 cells than in CEM-SS cells. Although our data suggest that differences exist in the sensitivities of cell lines to both the protective (tables 1 and 2) and direct cytotoxic (table 2) effects of test agents, the CEM-SS line has proven overall to be the most useful host cell line for identification of potential active compounds in the primary screen. Compounds that demonstrated antiviral activity in other cell lines have seldom had negative results in the CEM-SS cell line.

## Discussion

On the basis of the studies described here, we have implemented, as our high-flux primary anti-HIV-1 screen, the cell-free virus protocol (fig. 2B) using the HIV-1 (RF) virus with the CEM-SS host cell. If a test substance shows at least 30% protection against virus-induced cytopathic effects in the host cells, the microculture XTT antiviral assay is repeated. Additionally, at the termination of the assay, microscopic observations of cell survival are made, and aliquots are removed for correlative assays, such as the p24 antigen enzyme-linked immunosorbent assay (ELISA) and the syncytium assay for infectious virus.

The microculture XTT antiviral assay provides a quantitative representation of HIV-1-induced cytopathic effects as modulated by test substances. This relatively simple assay facilitates the safe and rapid determination of *in vitro* antiviral activity as well as direct cytotoxicity. We confirmed that this system measures infection and subsequent viral replication in target cells; we correlated XTT formazan production with the accumulation of extracellular virions, as measured by quantitative HIV-1-induced syncytium formation and p24 antigen synthesis. Our studies provided evidence for the validity of this assay. We performed double-blind studies in which AZT, an agent known to be clinically active, and other new prototype agents were reliably detected. Other potential target cell types, including both cultured and primary cells, such as peripheral blood lymphocytes and monocytes or macrophages,

are being evaluated for secondary assays to further characterize antiviral protection.

While the microculture XTT antiviral assay appears to be exceedingly useful for its intended application, there are nevertheless potential technical and theoretical limitations. For example, false-positive results may be generated by occurrence of the following problems individually or in combination: (a) nonspecific reduction of XTT by test compounds, (b) poorly understood drug-cell-XTT interactions, and (c) human error. Though it is a rare event, restoration of XTT formazan production (protection against viral cytopathic effects) without suppression of virus replication has also been observed in our laboratory. Thus, data generated by the XTT formazan-based drug screen may occasionally be subject to misinterpretation in the absence of confirmatory analyses. Useful correlative measures for confirmation of positive results in the primary screen are provided by cell-free drug controls, microscopic observation of all samples with positive results, and secondary tests (e.g., p24 or syncytium analysis) to validate suppression of virus replication.

Other laboratories (21,22) have recently reported the development of chromogen or cell viability dye-based assays and have proposed their use in screening for anti-HIV drugs. However, both of these assay systems require additional centrifugation or pipetting steps at the time of end-point determination. The study by Montefiore et al. (21), for example, requires suspension of drug-treated, virus-infected cells in microtiter trays and transfer of this suspension to a poly-L-lysine-treated microtiter tray, followed by incubation with neutral red, washing and centrifugation to remove excess dye, and extraction of the dye into acidified ethanol prior to colorimetric quantitation. An alternative microtiter-based MTT assay was recently suggested by Pauwels et al. (22); this assay also uses MT-2 cells as targets. The assay is also a potentially rapid screening method, but unlike the XTT system, it requires solubilization of the resulting formazan. Thus, both the MTT and neutral red assays necessitate additional centrifugation, pipetting, or aspiration steps with all of the attendant increased potential for aerosolization and exposure to HIV.

During 1988, the microculture XTT antiviral assay system was applied as a primary screen to the evaluation of >20,000 samples (10,000 plates), with use of both CEM-SS and MT-2 target cells for each test. The 20,000 samples included ≈7,000 unique compounds; 4,000 were pure synthetic or naturally occurring compounds, and 3,000 were crude extracts or partially purified fractions from natural products. A recent report by Vince et al. (23) described the first new synthetic anti-HIV-1 drug candidate identified by this screen; it is a novel carbocyclic nucleoside analogue. Moreover, since the pilot-scale implementation of the screen 1 year ago, ≈70 additional compounds (≈1% of unique samples screened) have shown *in vitro* anti-HIV-1 activity. Although a majority of the new active compounds are analogues of known or closely related classes of antiviral agents, a few entirely new nonnucleoside classes of antiviral compounds, including synthetic as well as natural products, have been identified. For example, Gustafson et al. (manuscript submitted for publication) have described a novel series of active anti-HIV sulfolipids



discovered by XTT anti-HIV bioassay, aided isolation from *Cyanobacteria* (blue-green algae). With the full implementation of the NCI anti-HIV-1 screening program, which is anticipated by mid-1989, we expect to achieve a screening capacity of at least 40,000 samples per year. The NCI high-flux primary anti-HIV-1 screen should therefore provide a powerful new drug-discovery support resource for AIDS-targeted efforts not only intramurally within NIH, but also, as intended, extramurally at national and international levels.

In conclusion, the microculture XTT antiviral assay provides an immediate solution to the need for large-scale primary screening facilities for potential antiviral compounds. The NCI continues to investigate suitable secondary screening systems as well as alternative new primary assays to operate in parallel with or even to replace this assay. It is hoped that the availability of this national resource will facilitate the prompt identification and development of new, clinically useful anti-HIV-1 drugs.

## References

- (1) MITSUYA H, WEINHOLD DJ, FURMAN PA, ET AL: 3'-Azido-3'-deoxythymidine (BW A509U): An antiviral agent that inhibits the infectivity and cytopathic effect of human T-lymphotropic virus type II/lymphadenopathy-associated virus in vitro. *Proc Natl Acad Sci USA* 82:7096-7100, 1985
- (2) MITSUYA H, MATSUKURA M, BRODER S: Rapid in vitro systems for assessing activity of agents against HTLV-III/LAV. In *AIDS: Modern Concepts and Therapeutic Challenges* (Broder S, ed). New York: Marcel Dekker, 1987, pp 303-333
- (3) DI MARZO VERONESE F, SARNGADHARAN MG, RAHMAN R, ET AL: Monoclonal antibodies specific for p24, the major core protein of human T-cell leukemia virus type III. *Proc Natl Acad Sci USA* 82:5199-5202, 1985
- (4) NARA PL, GERARD RW, MATTHEW GA, ET AL: Absence of cytotoxic antibody to human immunodeficiency virus-infected cells in humans and its induction in animals after infection or immunization with purified envelope glycoprotein gp120. *Proc Natl Acad Sci USA* 84:3797, 1987
- (5) POPOVIC M, SARNGADHARAN MG, READ E, ET AL: Detection, isolation, and continuous production of cytopathic retroviruses (HTLV-III) from patients with AIDS and pre-AIDS. *Science* 224:497-500, 1984
- (6) HARADA S, KOYANAGI Y, YAMAMOTO N: Infection of HTLV-III/LAV in HTLV-I carrying cells MT-2 and MT-4 and application in a plaque assay. *Science* 229:563-566, 1985
- (7) MITSUYA H, POPOVIC M, YARCHOAN R, ET AL: Suramin protection of T cells in vitro against infectivity and cytopathic effect of HTLV-III. *Science* 226:172-174, 1984
- (8) KARPAS A, GILLSON W, BLUM PC, ET AL: Lytic infection by British AIDS virus and development of rapid cell test for antiviral antibodies. *Lancet* 2:695-697, 1985
- (9) DALGLEISH AG, BEVERLEY PC, CLAPHAM PR, ET AL: The CD4 (T4) antigen is an essential component of the receptor for the AIDS retrovirus. *Nature* 312:763-767, 1984
- (10) NARA PL, HATCH WC, DUNLOP NM, ET AL: Simple, rapid, quantitative, syncytium forming microassay for the detection of human immunodeficiency virus neutralizing antibody. *AIDS Res Hum Retroviruses* 3:283-302, 1987
- (11) BOYD MR: Strategies for the identification of new agents for the treatment of AIDS: A national program to facilitate the discovery and preclinical development of new drug candidates for clinical evaluation. In *AIDS Etiology Diagnosis, Treatment and Prevention*, 1988 (DeVita VT Jr, Hellman S, Rosenberg SA, eds). Philadelphia: Lippincott, 1988, pp 305-317
- (12) HERRMANN EC, GABLIKS J JR, ENGLE C, ET AL: Agar diffusion method for detection and bioassay of antiviral antibiotics. *Proc Soc Exp Biol Med* 103:625-628, 1960
- (13) HUMBERT JR, MARKS MI, HAATHAWAY WE, ET AL: The histochemical nitroblue tetrazolium reduction test in differential diagnosis of acute infections. *Pediatrics* 48:259-267, 1971
- (14) MOSMANN T: Rapid colorimetric assay for cellular growth and survival application to proliferation and cytotoxicity assays. *J Immunol Methods* 65:55-63, 1983
- (15) SCUDERIO DA, SHOEMAKER RH, ALLEY MC, ET AL: Evaluation of a soluble tetrazolium/formazan assay for cell growth and drug sensitivity in culture using human and other tumor cell lines. *Cancer Res* 48:4827-4833, 1988
- (16) PAULL KD, SHOEMAKER RH, BOYD MR, ET AL: The synthesis of XTT—a new tetrazolium reagent that is bioreducible to a water-soluble formazan. *J Heterocyclic Chem* 25:911-914, 1988
- (17) MONTEFIORE DC, MITCHELL WM: Infection of the HTLV-II-bearing T-cell line C3 with HTLV-III/LAV is highly permissive and lytic. *Virology* 155:726-731, 1986
- (18) WILLIAMS GJ, COLBY CB: Combined effects of R interferon beta and AZT on HIV replication in vitro. *Antiviral Res* 9:138, 1988
- (19) ITO M, BABA M, SATO A, ET AL: Inhibitory effect of dextran sulfate and heparin on the replication of human immunodeficiency virus (HIV) in vitro. *Antiviral Res* 7:361-367, 1987
- (20) BABA M, PAUWELS R, BALZARINI J, ET AL: Mechanism of inhibitory effect of dextran sulfate and heparin on replication of human immunodeficiency virus in vitro. *Proc Natl Acad Sci USA* 85:6132-6136, 1988
- (21) MONTEFIORE DC, ROBINSON WE, SCHUFFMAN SS, ET AL: Evaluation of antiviral drugs and neutralizing antibodies to human immunodeficiency virus by a rapid and sensitive microtiter infection assay. *J Clin Microbiol* 26:231-235, 1988
- (22) PAUWELS R, BALZARINI J, BABA M, ET AL: Rapid and automated tetrazolium-based colorimetric assay for the detection of anti-HIV compounds. *J Virol Methods* 20:309-321, 1988
- (23) VINCE R, HUA M, BROWNELL J, ET AL: Potent and selective activity of a new carbocyclic nucleoside analog (carbovir: NSC 614846) against human immunodeficiency virus in vitro. *Biochem Biophys Res Commun* 156:1046-1053, 1988